Molecular Cloning and Characterization of Chitinase Gene *chiF* from *Streptomyces griseobrunneus* S3

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ABSTRACT

A preliminary survey of existence of family 19 chitinase gene among Taiwan native chitinolytic *Streptomyces* strains was conducted. Among 21 tested strains, 11 were detected positive by PCR (polymerase chain reaction) with the use of a family 19 chitinase gene specific primer pair. A full-length chitinase gene SGS3 *chiF* was cloned from *Streptomyces griseobrunneus* S3-a chitinolytic strain known to have great potential in plant disease control application. Sequence analysis revealed that the gene contains 891 bp which encodes a 296 amino acid peptide with a molecular weight around 31.5 KDa. The gene contains a chitin binding domain (ChtBD) with ChtBD consensus sequence AKWWTQ. On its upstream was a promoter region with characteristic direct repeat sequence (T/A) GGTC(T/C)AGACC(T/A) known to be critical in chitin induction and glucose repression. From a SGS3 *chiF* transformed *Escherichia coli* Rosetta™ (DE3), the chiF recombinant protein with a predicted size around 31.5 KDa was extracted and purified by Fast Performance Liquid Chromatography. The polyclonal antibody for immuno-detection of the recombinant protein was successfully prepared. The enzyme appeared to survive denaturing during SDS-PAGE analysis, the enzyme characteristics of recombinant protein was demonstrated by activity stain and western blotting. A phylogenetic analysis revealed that the cloned gene was highly homologous to the comparative gene known on *S. griseus*, *S. coelicolor*, and *Nocardiopsis prasina*, which might be adapted evolutionarily from the class IV chitinase of plant system. The molecular characteristics of the gene and its possible connection to antifungal characteristic of *Streptomyces* are herein discussed.

Key words: *chiF* gene, *Streptomyces griseobrunneus*, Family 19 chitinase.

INTRODUCTION

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosaminide (GlcNAc), is one of the most abundant polysaccharides in nature. It is a common constituent of insect exoskeleton, crustacean shell and fungal cell wall. The decomposition of chitin is important in the recycling of carbon and nitrogen resources in the biosphere. The presence of chitinase has been

implicated having critical importance physiologically and ecologically among bacteria, fungi, viruses, higher plants and animals. Chitinases known to date are classified into two categories- families 18 and 19, based on their polypeptide sequences and catalytic mechanisms (Armand *et al.*, 1994; Iseli *et al.*, 1996). The great majority of chitinases found to date are known to be members of family 18; whereas members of family 19 are unique in that they were found primarily in higher plants. *Streptomyces* are among micro-organisms the first known to possess family 19 chitinase (Ohno *et al.*, 1996; Saito *et al.*, 1999; Watanabe *et al.*, 1999).

In plant system, convincing evidences have demonstrated chitinases as one of the determinative factors contributing to active defence against the infection by plant-pathogenic microbes. The antifungal activity of class I, II and IV chitinases from various plant sources, especially those of family 19, has been well illustrated (Broekaert *et al.*, 1988; Iseli *et al.*, 1993; Leah *et al.*, 1991; Reborts and Selitrennikoff, 1988; Schlumbaum *et al.*, 1986). For family 19 chitinase recently discovered in *Streptomyces*, the role of the enzyme as an antifungal agent has been actively explored. A typical family 19 chitinase, chiC, discovered from *S. griseus* HUT6037 strain, was shown having anti-fungal activity on the agar plate against *Trichoderma reesei* (Watanabe *et al.*, 1999). And the *chiC* transgenic rice was shown having increased resistance against infection by *Magnaporthe grisea* (Itoh *et al.*, 2003).

In an attempt to explore microbial resources for biopesticide development, *Streptomyces griseobrunneus* S3 (SGS3) was selected from cohorts of rhizosphere microbes for its superior chitinolytic activity and antagonistic effect against phytopathogenic fungi. By a dual culture assay, the bacterium was found strongly antagonistic against *Pythium aphanidermatum*, *Phytophthora parasitica*, *Colletotrichum gloeosporioides*, *Guignardia citricarpa*, *Pyricularia oryzae* and *Rhizoctonia solani* AG4 (Lai, 2003). It's potential as a biofungicide for plant disease control has been demonstrated, and the disease control efficacy was shown due at least in part to the mycoparasitic activity. The functioning of chitinolytic activity appeared to be critical in the anti-fungal activity observed. In order to elucidate the molecular basis of the biocontrol efficacy, the possible involvement of chitinase genes of this bacterium was investigated. A family 19 chitinase gene *chiF* was shown in an *in vitro* culture system among the surveyed chitinase genes preferentially expressed when colloidal chitin was applied. The full-length SGS3 *chiF* was cloned, and the characteristics of the gene expression and the enzyme cleavage activity were further investigated. Also, the existence of *chiF* gene among various chitinolytic antagonistic bacterial/fungal strains was surveyed. The molecular and biochemical characteristics of the gene and the encoded enzyme protein are herein discussed.

MATERIALS AND METHODS

Microbial strains culturing and preservation

A total of 21 *Streptomyces* strains shown in Table 1 were used for this investigation. All the test strains had been shown to have superior chitinolytic characteristics in our previous works (Lai, 2003). Except S16 and SS31 which were kindly provided by Prof. Jenn-Wen Huang and Dr. Tung-Tsuan Tsai, each respectively, all other tested strains were isolated from rhizosphere in a serial

screening works aiming for chitinolytic antagonists with potential in plant protection application.

All the bacterial strains were cultured on potato sucrose agar (PSA) plate at 30℃ in dark for 3 days. The bacterial spores/ mycelia were then harvested and suspended in sterilized distilled water containing 0.05% (v/v) Tween 20 and stored at 4℃ for long term preservation. The tested fungal strains were cultured on PSA plate at 30℃ for 5 days, and then stored at 4℃. For experiments conducted, the microbes were allowed to grow on PSA plate as above indicated. And the freshly prepared spores/mycelia suspensions were then used as inoculum for the experiments.

Extraction of total DNA from *Streptomyces*

Genomic DNAs were extracted from *Streptomyces* species by the method described by Kutchma et al. (1998). The test bacteria were cultured in 20 ml potato sucrose broth in 250 ml flask under continuous shaking at 120 rpm. The mycelia were collected by 10 000×*g* (SS-34 rotor, Sorvall® RC5C) centrifugation at 4 ℃ from overnight culture, suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7) containing 1 mg/ml lysozyme (Amresco Chemical) and incubated at 37℃ for 1-2 h or until the suspension turn slightly viscous due to partial degradation of cell wall. Sodium dodecyl sulfate (SDS) and NaCl were then added to final concentration at 1% and 1 M, respectively, and the sample was then subjected to repeated freeze/thawing for 3 times. After removing cell debris by centrifugation, the DNAs were purified by phenol/chloroform extraction and ethanol precipitation. The genomic DNAs obtained were then used for PCR and southern analysis.

Table 1. *Streptomyces* strains used in this study.

Strains	Sources
S1 (*Streptomyces* sp.)	Peat moss, Soil Mix, HEVECO, Canada.
S2 (*Streptomyces* sp.)	Peat moss, Floral Guard, TKS, Germany.
SGS3 (*S. griseobrunneus*)	Citrus rhizosphere, Taiping, Taichung County.
S4 (*Streptomyces* sp.)	Peat moss, Soil Mix, HEVECO, Canada.
S5 (*Streptomyces* sp.)	Taro rhizosphere, Dali, Taichung County.
SL7-1 (*S. lincolnensis*)	Sesbania rhizosphere, Hsingkang, Chayi County.
SL7-2 (*S. lincolnensis*)	Sesbania rhizosphere, Hsingkang, Chayi County.
S8 (*Streptomyces* sp.)	Taiwan Anoectochilus rhizosphere, Taichung.
S9 (*S. fimbriatus*)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S10 (*Streptomyces* sp.)	Grape rhizosphere, NCHU, Taichung.
S12 (*Streptomyces* sp.)	Sesbania rhizosphere, Hsingkang, Chayi County.
S14 (*Streptomyces* sp.)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S15 (*Streptomyces* sp.)	Soil, Datsun, Changhwa County.
S16 (*Streptomyces* sp.)	Mushroom growth substrate, Sinshe, Taichung County.
S18 (*Streptomyces* sp.)	Sugarcane rhizosphere, Hsingkang, Chayi County.
S20 (*Streptomyces* sp.)	Citrus rhizosphere, Taiping, Taichung County.
SS31 (*S. saraceticus*)	Dead cicada remain in citrus orchard.
S38 (*Streptomyces* sp.)	Peanut rhizosphere, Taiping, Taichung County.
SC15 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.
SC17 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.
SC19 (*Streptomyces* sp.)	Soil, Wufeng, Taichung County.

† Strain S31and S16 were each respectively kindly provided by Prof. Jenn-Wen Huang and Dr. Tung-Tsuan Tsai, Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan.

Molecular cloning and detection of the expression of partial *chiF* from SGS3

Partial sequence of *chiF* was amplified by PCR extension by use of specific primer pair shown below:

ChiFF	5′AAG CGC GAG GCC GCG GCC
ChiFR	5′GCA CTC CAG AGC GCC GTT GAT 3′

The primers designed for *chiF* was based on the nucleotide sequence of *chiC* (accession number AB009289) of *S. griseus* (Watanabe *et al.*, 1999) in corresponding to the amino acid sequences from Lys-134 to Ala-142 and from Ile-256 to Cys-262, respectively. With the addition of SGS3 genomic DNA as template, PCR amplification was performed for 30 cycles by the scheme at 94℃ denaturation for 30 sec, 62℃ reannealing for 30 sec, and 72℃ extension for 30 sec; and a final cycle at 72℃ for 5 min. To avoid the formation of DNA secondary structure due to high GC content of *Streptomyces* genome, 1% dimethyl sulfoxide (DMSO) and 1 M betaine were amended in PCR reaction buffer (Baskaran *et al.* 1996). The amplicons were then cloned with TA-cloning kit (yT&A TA cloning kit, Yeastern Biotech Co., Taipei, Taiwan). For southern and northern analysis, digoxigenin (DIG) was incorporated into these cloned sequences according to the manufacturer's protocol (DIG DNA labeling kit, Roche, Mannheim, Germany).

Preparation of colloidal chitin and glycol chitin

Colloidal chitin was prepared by the method described by Lingappa and Lockwood (1962) using powdered crab shell chitin purchased from Sigma (St. Louis, MO, USA). The chitin powder was digested with 50% sulfuric acid for 10 min, washed with distilled water several times until pH reached 6-7, and then diluted to 2% (w/v) with distilled water for medium preparation. Glycol chitin was prepared by the method described by Yamaga and Imoto (1981). Approximately 1 g of glycol chitosan (Sigma, St. Louis, MO, USA) was immersed in 20 ml of 10% acetic acid overnight, washed with 90 ml methanol, reacted with 1.5 ml of acetate anhydride under continuous stirring at room temperature for 30 min, and then washed again with methanol for several times. The glycol chitin thus obtained was then precipitated by centrifugation at 5000 × *g* (SS-34 rotor, Sorvall® RC5C), suspended in 0.02% (w/v) sodium azide and stored at 4℃ until use.

Detection of *chiF* among tested *Streptomyces* strains

Genomic DNAs of all 21 tested *Streptomyces* strains (listed in Table 1) were extracted by the method described above; the existence of *chiF* was detected by PCR amplification using the primer pair *chiF*F/*chiF*R. The PCR amplification scheme was same as that above described.

Cloning of *chiF* from SGS3

The genomic library of SGS3 was constructed by use of ZAP Express® Predigested Vector Kit and ZAP Express® Predigested Gigapack Cloning Kits (Stratagene, CA, USA). Genomic DNA was restriction digested with *Bam*HI and resolved by agarose gel electrophoresis. The resolved fragments near 3 kb were recovered and ligated to the ZAP express vector according to the manufacturer's protocol. The *chiF* recombinants were screened by

southern analysis using the DIG-labeled partial *chiF* fragment as detection probe, the contained insert was then fully sequenced.

Construction of *chiF* expression vector and purification of the recombinant protein

For construction of the expression vector, the coding region of *chiF* was amplified by PCR with the use of a forward primer (5′-GATCCATGGTGTTCAAACGTGTCCTC-3′) which contains sequence identical to N-terminal of a *Nco*I site (sequence underlined) inserted *chiF* gene flanking at its 5′ end, and a reverse primer (5′-GATGAATTCCCGCAGCTCAGGTTCG A-3′) which contains sequence complementary to the C-terminal of an *Eco*RI site (sequence underlined) inserted *chiF* flanking at its 5′ end. As that shown in Fig. 1., the amplicon was then ligated to a *Nco*I- and *Eco*RI- digested plasmid vector pET26b (Novagen, EMB Biosciences Inc., Germany) following the manufacturer's protocol.

The constructed plasmids were then used to transform competent cells of *E. coli* Rosetta™ (DE3), and the recombinant strains which harbor the expression plasmid pET26b/*chiF* were selected and allowed to grow at 37℃ in LB medium containing kanamycin (100 μg/mL). The suspended cell cultures were fed with 1 mM of IPTG when the A_{600} of the cell suspension reached 1.0, the cells were then allowed to grow for another 4 hr at 28℃. The cells were then pelleted by centrifugation (10 000 × *g*, GSA rotor, Sorvall® RC5C), and the recombinant chiF protein which exist predominantly in periplasmic fraction was extracted by an osmotic shock facilitated protocol (manufacturer provided) and concentrated by ammonium sulfate precipitation. The recombinant proteins obtained were dissolved in 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and resolved by a ÄKTA FPLC system with the use of Superdex 200 HR 10/30 column (Amersham Biosciences, Cardiff, UK). The samples were eluted with the same buffer at 0.5 ml/min flow rate, and desalted by an Amicon® Ultra-15 centrifugal filter device (Millipore, Billerica, Mass.,USA) where 20 mM phosphate buffer (pH 7.0) was applied as equilibrating buffer.

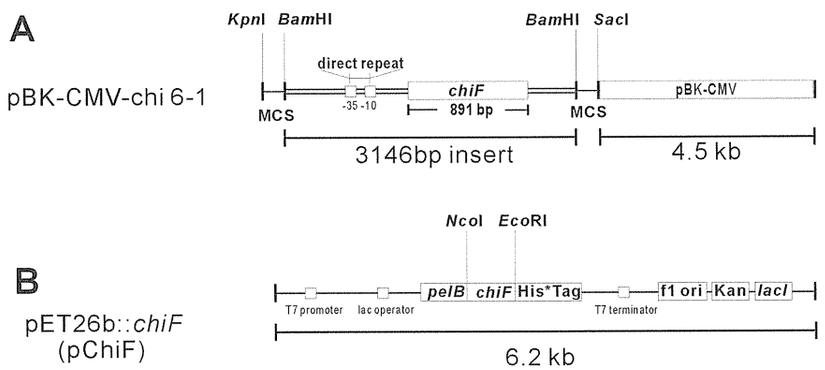

Fig. 1. Construction scheme of cloning vector pBK-CMV-chi 6-1(A) and SGS3 *chiF* overexpression vector pChiF (B).

Detection of chitinase activity in SDS-PAGE with glyco chitin amendment

SDS-PAGE was conducted as that described by Bollag *et al*. (1996) except that the acrylamide gel was amended with 0.01% glycol chitin. The electrophoresis resolved gel was washed with renaturing buffer [consists of 0.1 M sodium acetate, 1% (w/v) triton X-100, pH 5.0] for 20 min twice and then stained with 0.01% (w/v) Calcofluor White M2R (Sigma, St. Louis, MO, USA) (dissolved in 0.5 M Tris-HCl, pH 8.9). It was then destained with distilled water and the chitinolytic band was visualized with UV light.

Preparation of chiF polyclonal antiserum

The recombinant chiF protein obtained from ÄKTA FPLC purification was used to immunize New Zealand white rabbit. The protein samples were adjusted to 2.0 mg/L in concentration by the addition of 20 mM sodium acetate buffer (pH 5.8). Approximately 1 mg of the sample was injected on a weekly basis. The first injection was applied with the addition of equal volume of Freund's complete adjuvant (Difco Laboratories Detroit, Michigan), whereas the followed three injections were done with the addition of Freund's incomplete adjuvant. The rabbit was bled 1 week after the last injection. The antiserum obtained was dispensed in eppendorf tubes and stored at -20℃ for further application.

Detection of chiF by western blotting

The chiF protein resolved by SDS-PAGE was electro trans-blotted onto nitrocellulose membrane by use of BioRad transblot apparatus (Bio-Rad, Hercules, CA). The western blot analysis was performed by the protocol described by Sambrook *et al*., (1982) using chiF polyclonal antibody prepared.

Phylogenetic analysis of *chiF* clonned

The 145-257th amino acid sequence (113 amino acids in length) as deduced from the catalytic domain of *chiF* was used for the analysis performed. The comparative amino acid sequences- with the lengths ranging from 102-137 amino acids, which were available on gene bank of NABI known from a total of 29 different biological origins (Table 2) were used for the analysis.

The analysis was conducted with the use of DNASTAR sequence analysis software (DNASTAR, Madison, USA). The amino acid sequences alignment was processed by MegAlin program of DNASTAR by the method of Clustal W.; the neighbor- joining method was used for the sequence comparison and the construction of the phylogenetic tree.

RESULTS

Molecular cloning and detection of *chiF* expression in SGS3

With the application of the designed primer pair and the use of SGS3 genomic DNA as template, the partial sequence of *chiF* was amplified and cloned. The size of cloned *chiF* was 387 bp. The cloned partial gene in accordance with each respective corresponding chitinase gene on *S. griseus* and *S. coelicolor* were illustrated on Fig. 2.

Detection of *chiF* among tested *Streptomyces* strains

The PCR amplification using *chiF*F /*chiF*R as primer pair indicated that eleven test strains including S1, SGS3, S4, S5, SL7-1, SL7-2, S9, S10, S12, S14, and S38 were detected positively with *chiF* on their

genomic DNAs (Fig. 3). The rest 10 test strains, however, showed negative responses in the PCR amplification conducted.

Table 2. Chitinase peptide sequences used for comparison in this study.

Accession Nos.	Source organisms	Protein ID.
AAA56787	Barley	chitinase, class I
AAA51377	Rice	chitinase, class I
AAA32769	Thale cress	chitinase, class I
CAA87072	European elder	chitinase, class I
CAA33517	Potato	chitinase, class I
CAA34812	Tobacco	chitinase, class I
CAA39535	Rice	chitinase, class I
AAA34107	Tobacco	chitinase, class II
CAA47921	Potato	chitinase, class II
CAA35791	Petunia	chitinase, class II
AAA32941	Barley	chitinase, class II
CAA40474	Kidney bean	chitinase, class IV
AAA32916	Beet	chitinase, class IV
CAA43708	Rape	chitinase, class IV
AAA33444	Maize	chitinase, class IV
BAA84194	*S. coelicolor*	Partial family 19 chitinase
BAA84195	*S. lividans*	Partial family 19 chitinase, f1
BAA84196	*S. lividans*	Partial family 19 chitinase, f2
BAA84197	*S. lividans*	Partial family 19 chitinase, f3
BAA84199	*S. coelescens*	Partial family 19 chitinase
BAA84200	*S. ipomoeae*	Partial family 19 chitinase, f1
BAA84201	*S. prasinopilosus*	Partial family 19 chitinase, f1
BAA84202	*S. prasinopilosus*	Partial family 19 chitinase, f2
BAA84203	*Streptomyces* sp. Strain S15	Partial family 19 chitinase
BAA84204	*Streptomyces* sp. Strain S84	Partial family 19 chitinase
BAA84205	*Streptomyces* sp. Strain S100	Partial family 19 chitinase
BAA84206	*Streptomyces* sp. Strain S159	Partial family 19 chitinase
BAA23739	*S. griseus*	Complete family 19 chitinase,
AY953370	*S. lincolnensis* (SLS7)	Partial family 19 chitinase

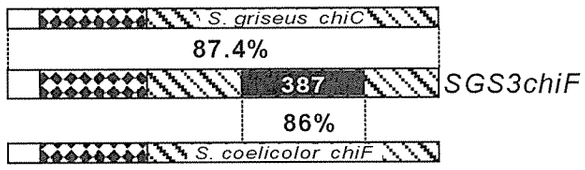

Fig. 2. An illustration (modified from Saito et al. 2000) showing sequence identity of the cloned SGS3 *chiF* comparing to each of the corresponding genes known in *S. griseus* and *S. coelicolor*. The black box indicated the partial *chiF* cloned, the size (bp) was printed in white on black box. Numbers between the regions of the gene show the percentage of sequence identity with compared gene fragment. Open boxes, signal sequence; checkered boxes, substrate-binding domain; and striped boxes, catalytic domain.

The detection by northern analysis of chitinase gene expression in SGS3 grown in colloidal chitin (0.01%) amended Czapek Dox Broth indicated that the expression of *chiF* appeared to be transient which reached a maximum level generally 2-4 h after addition of colloidal chitin and decreased thereafter (data not shown). The expression *chiF* was greatly repressed, however, without colloidal chitin amendment or when glucose was added as the only or the additional carbon source (data not shown). An adjunct Blast analysis indicated that *chiF* belongs to the member of family 19 chitinase, which has been implicated to be critical in chitin degradation and antifungal activity of members of *Streptomyces* (Watanabe *et al.*, 1999).

Cloning of full-length *chiF* gene from SGS3

From the genomic library constructed, a total of 42 *chiF* positive phage clones were selected by southern analysis. Among them, clone chi 6-1 that harbors an insert of 3146 bp (Fig. 4) was arbitrarily selected and fully sequenced.

The computer analysis with Seq Web Version 2 software (adapted from http://apdb1.nhri.org.tw:8003/gcg-bin/seq web.cgi, Accelrys, Inc.) indicated that the insert contains only one open reading frame of 891 bp in size (Fig. 5). The gene contains a secretory signal peptide domain, a chitin binding domain (ChtBD) and a catalytic domain. The open reading frame encoded a polypeptide of 296 amino acid residues and a predicted polypeptide at molecular weight of 31.5 kDa. A ChtBD consensus sequence AKWWTQ (Tsujibo *et al.*, 2003) was found at the 24-29th amino acids of the deduced peptide sequence. A possible promoter sequence TTGAGT (-10, single underlined) and TTGACA (-35, double underlined) were found at 62 and 86 upstream of the translation site (Figs. 5 and 6). And in adjacent to each of them was the direct repeat sequence TGGTCTAGACCT and AGGTCCAGACCA (Delic et al. 1992; Ni and Westpheling 1997). The cloned *chiF* gene was 87.4% identical to *S. griseus chiC*. The resolved *chiF* nucleotide sequence has been submitted for registration by National Center for Biotechnology Information and is now available by the accession number AY348315.

Production and purification of SGS3 chiF from *E. coli*

From broth culture of *E. coli* Rosetta™ (DE3) that harbors the transformed pET26b/*chiF*, production of chiF protein was achieved by IPTG induction. The chiF protein extracted was concentrated by ammonium sulfate (at 90% saturation) precipitation, resuspended with phosphate buffer (20 mM, pH 7), and resolved by chelating gel on an ÄKTA FPLC system. A protein peak (arrow indicated) with expected enzymatic activity was detected (Fig. 7).

A SDS-PAGE analysis indicated the recombinant protein has molecular weight around 31.5 kDa (Fig. 8A lanes 1 and 2). The followed western blot analysis further revealed that the detected protein band reacted positively with chiF antibody prepared (Fig. 8B). The chitinolytic activity of the major band was demonstrated by fluorescent staining with Calcofluor white M2R (Fig. 8C) and as well by development of clear chitinolytic zone on the colloidal chitin amended agar plate (Fig. 8D).

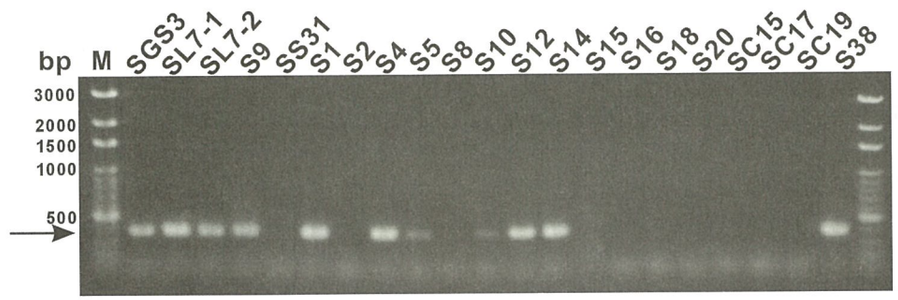

Fig. 3. PCR amplification of family 19 chitinase from 21 *Streptomyces* spp. strains by using primer pair *chi*FF/*chi*FR. The arrow indicated the predicted 387 bp amplicon of the partial family 19 chitinase gene.

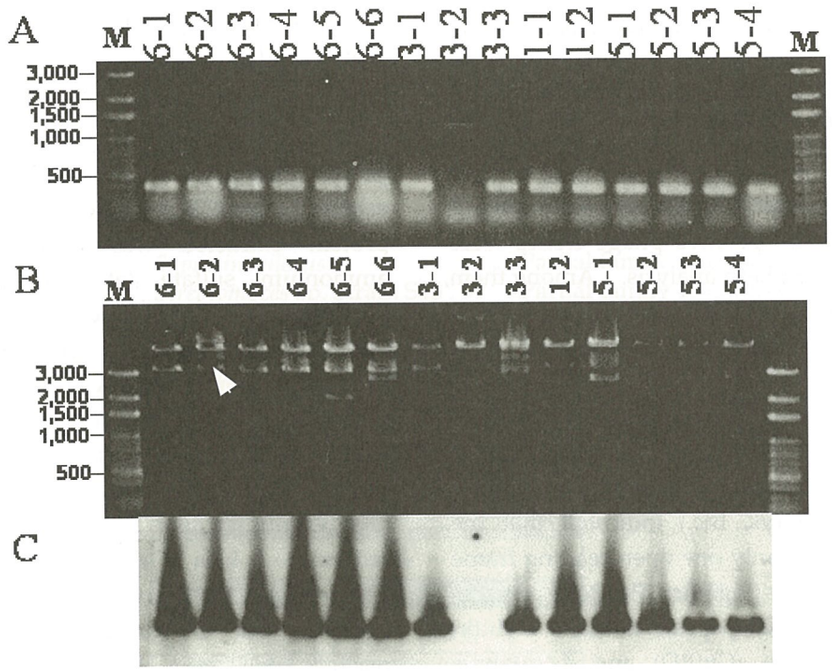

Fig. 4. Detection of *ChiF* gene among the transformed *E. coli* isolates obtained. The *ChiF* gene was detected through PCR amplification using primer set ChiFF/ChiFR(A). The phagemid DNAs from each isolates were digested with *Bam*HI and resolved by 1% agarose(B) and subsequently hybridized with *ChiF* probe (C). The arrow designated the target 3kb DNA fragment containing *ChiF* gene. Lane M, 100 bp DNA ladder marker (GeneMark).


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1    ggatccggtc cccgccgagg ttctccgcga ggtggtacga ggcccgtgtg ccgggcaccg gcaccacgtg gtcaccgagc cggagcagcc atgccagggc
101  gacctgcgcg ggggtggcct cgtgccgggc cgccacctgg gcgacggcct gcgtgagcgc gtagttgtgg cggatggccc gcgggggcgaa gcgcggctgg
201  gtgcgccgcc agtcgtccgg ggccaggtcc tcggtgcggg agtacgcgcc ggtcagcagc ccgcgcccga gcggggcacc ggccagcagg gcgatccggc
301  gctccaccgc gtacggcacg acgtcgtcga ggccgcaccg cgtgaacagc gacacctcgg cgccgatcgc cgccgcctcg tgcacggcct cggcctcgcg
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701  ctccccgtcc gcgtaggcgt cggccgtctc cagcaggttg accccgaggt cgaccgcctc ctggacgagc gacaccgcct tggtccggtc caccaggccg
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1401 cac gtg ttc aaa cgt gtc ctc agc ctg ctg gtc gcg ctc ggc gcg gtc gtc gcg gcg ttc gtc gtc ctc ccc gcc gcc acc gcg
        M  F  K  R  V  L  S  L  L  V  A  L  G  A  V  V  A  A  F  V  V  L  P  A  A  T  A
1485 cag gcc gcc acc tgc ttc agc gcc tgg aac tcc tcg tcc gtc tac acc ggc ggc cag acc gcc tcg tac aac ggc cgg aac tac
     Q  A  A  T  C  F  S  A  W  N  S  S  S  V  Y  T  G  G  Q  T  A  S  Y  N  G  R  N  Y
1569 acc gcc aag tgg tgg acc cag aac gag cgt ccg ggc tcc tcc gac gtc tgg acc gac aac ggc gcc tgc ggc acg ggc ggc ggc
     T  A  K  W  W  T  Q  N  E  R  P  G  S  S  D  V  W  T  D  N  G  A  C  G  T  G  G  G
1653 ggt gag gac ccg ggc cag agc aac ggg ttc gtc gtc agc gag gcg cag ttc aac cag atg ttc ccg aac cgg aac tcg ttc tac
     G  E  D  P  G  Q  S  N  G  F  V  V  S  E  A  Q  F  N  Q  M  F  P  N  R  N  S  F  Y
1737 acg tac cgg ggt ctc acc gac gcc ctg agc gcg tac ccc gcc ttc gcc aac acc ggc agc gac gag atc aag aag cgc gag gcc
     T  Y  R  G  L  T  D  A  L  S  A  Y  P  A  F  A  N  T  G  S  D  E  I  K  K  R  E  A
1821 gcg gcc ttc ctc gcc aac gtc agc cac gag acc ggc ggg ctg gtg tac atc gag gaa gtc aac gag gcc aac tac ccc cac tac
     A  A  F  L  A  N  V  S  H  E  T  G  G  L  V  Y  I  E  E  V  N  E  A  N  Y  P  H  Y
1905 tgc gac gcc agc cag ccc tac ggc tgc ccc gcc ggc cag tcc gcg tac tac ggc aag ggc ccg atc cag ctg agc tgg aac ttc
     C  D  A  S  Q  P  Y  G  C  P  A  G  Q  S  A  Y  Y  G  K  G  P  I  Q  L  S  W  N  F
1989 aac tac aag gcc gcc ggt gac gcg ctc ggc atc gac ctg ctg aac aac ccc tac ctg gtc gag cag aac gcc gcc gtc gcc tgg
     N  Y  K  A  A  G  D  A  L  G  I  D  L  L  N  N  P  Y  L  V  E  Q  N  A  A  V  A  W
2073 aag acc gcc ctc tgg tac tgg aac acc cag acc ggc ccc ggc acc atc acg ggc cac aac gcc atc gtc aac ggc ccc ggc ttc
     K  T  A  L  W  Y  W  N  T  Q  T  G  P  G  T  I  T  G  H  N  A  I  V  N  G  P  G  F
2157 ggc gag acg atc cgc tcc atc aac ggc gcg atc gag tgc aac ggc ggc aac ccg gcc cag gtc cag agc cgc atc gac aag ttc
     G  E  T  I  R  S  I  N  G  A  I  E  C  N  G  G  N  P  A  Q  V  Q  S  R  I  D  K  F
2241 acg cag ttc acg cag gtt ctc ggc acc acg acc ggg tcg aac ctg agc tgc tga ttccgg gcgcgttcca cgaacgtcga acgttccccg
     T  Q  F  T  Q  V  L  G  T  T  T  G  S  N  L  S  C  *
2331 cgcgacccgt cccgtacggg ggtccgcccg gctgctgccg ggagggcccc cgttccggcg ttccggccgt tccggcgttc cgggagacgg tcggcaggat
2431 cgggtacgtc aggccagcgg cggcttcacc gacatcagca gatgccggtg gcggggaggg gagacgtccc ggccgggttc accctcgccc gtgatcatcg
2531 cccgctgccc cgcccccatc atcagcagcc gcagcgacgg ctcgtcgaag gaggccaggg cgtccgccag atacgccggg ttgaacgcca cggtcatctc
2631 gcgggcgcct tccagggtcg cgggcaaccg ctgggaggcc acgtcgtcct cgtaaccggc tgcagatgta cggagttgtc gtcggcggag aacgccaatt
2731 ggaccggccc gtcccgtcgg cgaccaccga gacccgcttg acggcctcca ccagccgcgc ccggtccacc accgccactg ccgggtcctc catcgcgaag
2831 agcttgttgt ggcgcgggag ccgtccgtcg agcagtcgca cggtggtccg catcccgcgg tgctcgaacc ccgccgaacc ggcgcccagg gcgacgctca
2931 ccagcccgga ccgccccagc gaacgggcga tctcggtcaa ccgccgcgcg gagaccagca catcggcact ttcaccggcc tcggaccctt cacccgcccc
3031 cgtatccgca cccgccccgg ccgccttcca aggcaacgtc cgtacggcga accggtaccg gtcggtcgcg gcgagcgtca tcgtccccgt cccgcgtcg
3131 agccccagcc ggatcc
```

Fig. 5. Nucleotide sequence and deduced amino acid sequence of SGS3 *ChiF* gene of *S. griseobrunneus* S3. Amino acids are specified in single-letter code under the first of each codon. The -35 and -10 sequence of each promoter are double- and single-underlined, respectively. Shadow box and open box indicated the secretory signal peptide and chitin binding domain (ChtBD), respectively. Catalytic domain is printed in white on black boxes. It consists 891 bp in length, which encodes a 296 amino acid peptide with a molecular weight of 31.5 KDa. A ChtBD consensus sequence AKWWTQ (shown in bold type) was found in the gene at 24-29th amino acids of the peptide sequence.

p-*chiF* gaacgcaagttgaacTGGTCTACACCCttgacTGGTACAGACCAaagcggttgagtatgcctccacacc

SGS3 *ChiF* ccgacgtcacgaaggaTGGTCTAGACCTtgacAGGTCCAGACCAtcatcgcttgagtgtcggcaccccc

Consensus TGGTC(C/T)(A/G)GACC(A/T)

Fig. 6. Nucleotide sequence of the promoter regions of *chiF* gene (p-*chiF*) of *S. coelicolor* A3(2) and *ChiF* gene of *S. griesobrunneus* S3. The –35 and –10 sequence of each promoter are double- and single-underlined, respectively. Nucleotide sequences of direct repeats are printed in upper-case letters and those identical to the consensus sequence (Saito *et al.* 2000) are printed in white on black background.

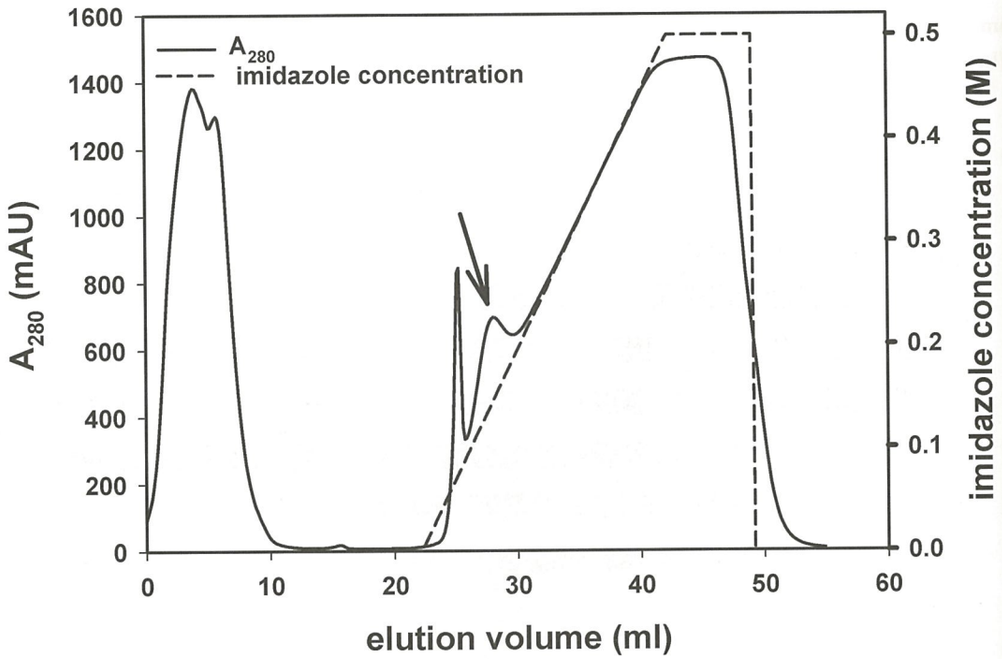

Fig. 7. Purification of chiF protein by chelating gel on an ÄKTA FPLC system. Ammonium sulfate precipitated SGS3 chiF protein was dissolved in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4) and applied onto HiTrap Chelating HP column. The arrow indicates SGS3 chiF protein peak eluted.

Phylogenetic correlation of *chiF* among the known Family 19 chitinase genes

The pileup alignment of the catalytic domain family 19 chitinase amino acid sequences known from 15 *Streptomyces* strains comparing to that from 15 plant resources indicated clearly the existence of high sequence identity among the *Streptomyces* strains analyzed (Fig. 9). The high sequence identity was shown further by the phylogenetic tree analysis where that the 15 test *Streptomyces* derived chitinases were sorted into one group which appeared to be distinct from all the compared plant derived sequences (Fig. 10). The alignment analysis also indicates the close relatedness among these *Streptomyces* enzymes and the compared plant derived enzymes, the identical sequences was detected mainly from the region ranging from the 60-110th amino acids (Fig. 9). What worth noting was that these enzymes appeared to share high similarity to all the plant Class IV chitinase tested including that from European elder, kidney bean, rape, beet and maize, in that there were deletion sequences at 33-50th and 111-131st amino acid sequence region. In contrast to this, the deletion regions were not detected among the plant derived Class I and Class II enzymes tested (Fig. 9).

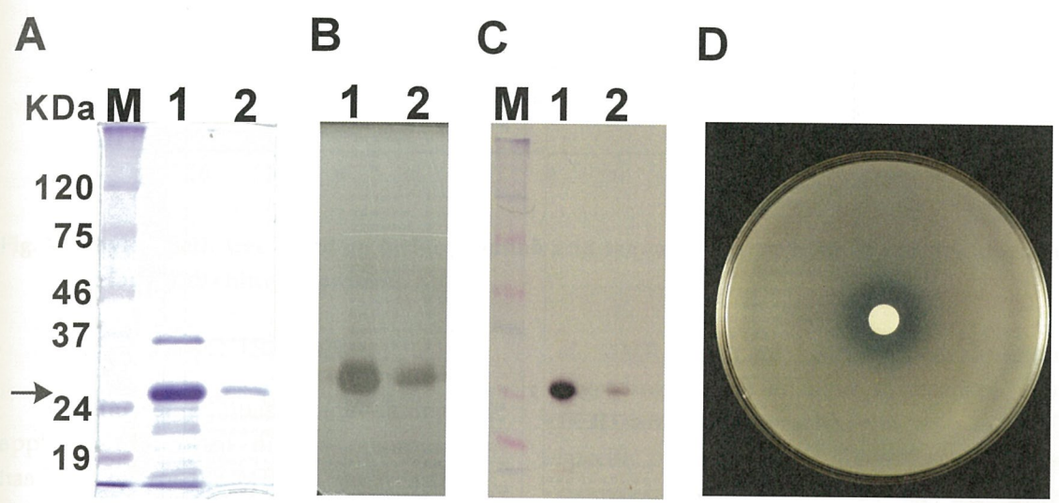

Fig. 8. Detection of recombinant SGS3 chiF protein by SDS-PAGE (A and B), western blot (C), and by clear zone formation on 0.5% colloidal chitin amended agar plate (D). Recombinant chiF was extracted by ammonium sulfate precipitation and purified by chelating gel chromatography. For SDS-PAGE analysis, approximately 10 μg of the protein sample were loaded on each lane, and the resolved gel was stained with Coomassie blue (A) or with Calcofluor white M2R under UV illumination (B). Lane M, molecular weight marker (ProSive® color protein markers, BioWhittaker Molecular Applications Inc., USA). Lane 1, sample from ammonium sulfate precipitation; lane 2, sample purified from chelating gel chromatography. The arrow indicates the recombinant chiF protein with the molecular weight at 31.5 KDa. The proteins were trans-blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and reacted with the chiF antiserum. The existence of chiF was revealed by indirect enzyme-linked immunoassay (C) by use of phosphatase labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) as secondary antibody. For the plate assay (D), approximately 100 μg of the purified enzyme were applied on the paper disc laid on 0.5% colloidal chitin containing agar. The picture shown was taken 2 days after treatment.

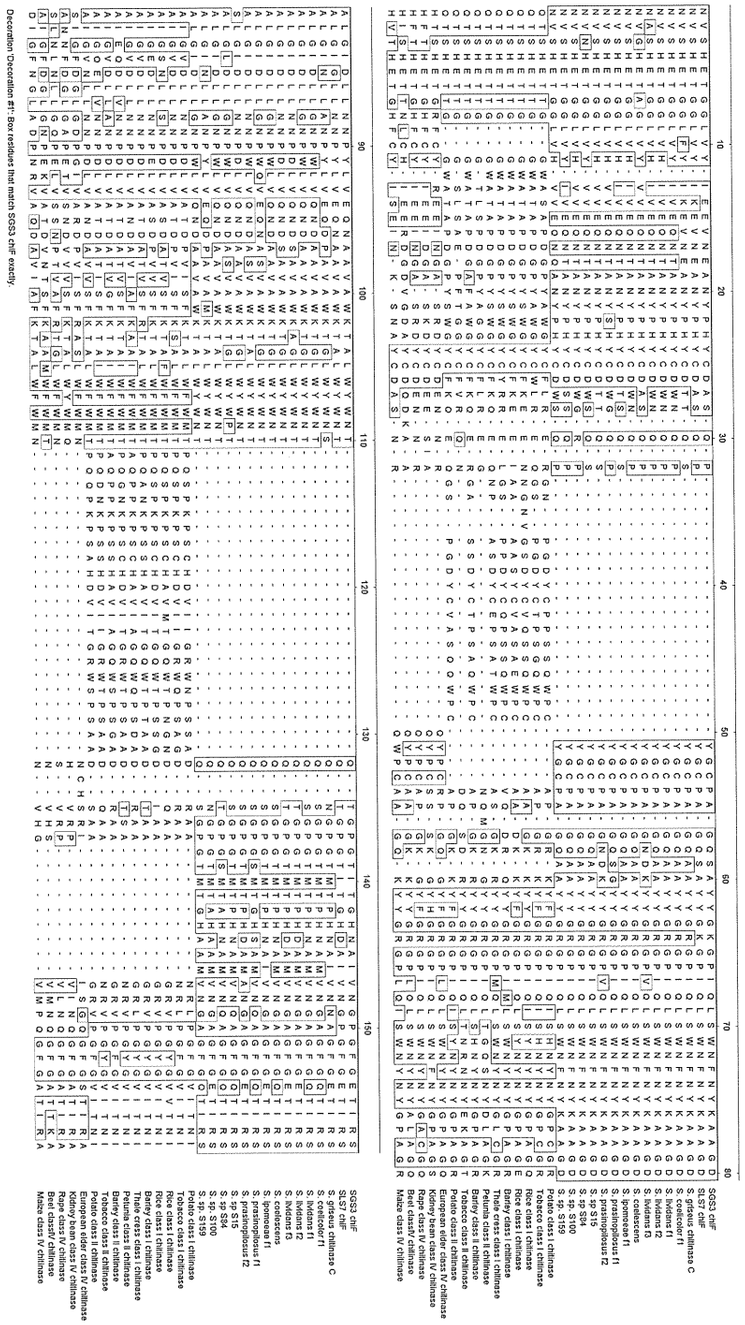

Fig. 9. Pileup alignment of family 19 chitinase genes. Amino acid sequences alignment were processed by MegAlin program of DNASTAR sequence analysis software (DNASTAR, USA) by the method of Clustal W. "-" indicated the amino acid sequence deletion site (gap). The sequences identical to SGS3 chiF were surrounded with boxes.

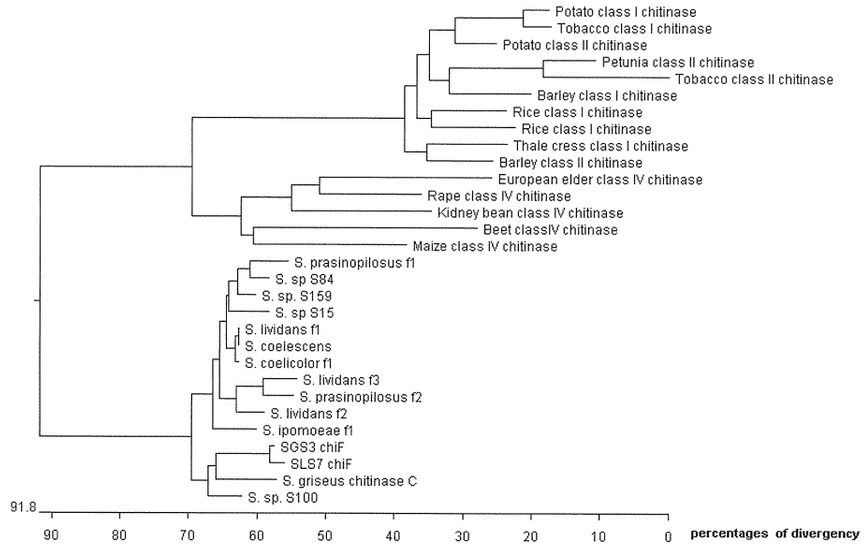

Fig. 10. Phylogenetic tree based on deduced amino acid sequence comparisons of catalytic domain of family 19 chitinase protein.

DISCUSSION

Cloning of chitinase gene aiming for application in plant disease manipulation has been one of the primed research topics during the past decade. The family 19 chitinase, initially discovered exclusively from plant system was known to be a major constituent of pathogenesis related protein (PR protein) with critical importance in induced resistance. Their antifungal characteristics have been well illustrated (Tsujibo *et al.*, 2003). The development of antagonistic *Streptomyces* as bio-fungicide was gaining great attention lately because the difficulty of disease control and the concern on the deleterious impact resulted from chemical pesticide application. The initiative discovery of family 19 chitinase gene *chiC* among members of *Streptomyces* (Saito *et al.*, 2000) and the detection from there-of prepared transgenic rice the enhanced rice blast disease resistance (Itoh *et al.*, 2003) prompted further the exploration regarding to the enzyme application. By PCR amplification with the use of *chiF* specific primer pair, the above shown results demonstrated the presence of the gene among most of the tested antagonistic *Streptomyces* strains (Fig. 3). The existence of *chiF* homologous sequence among these tested strains indicated its popularity among the Taiwan native strains. The failure to detect the homologous sequence from 10 of the tested strains appeared due to the divergence of the gene rather than the absence of the target gene; an adjunct western blot analysis has shown the existence of the enzyme among most of these test strains (data not shown).

For the 891bp full-length sequence of *chiF* cloned, the pair-wise sequence analysis indicated that it shared 87.4, 85, and 73% identity, respectively, comparing to that of *S. griseus chiC*, *N. prasina chiB*, and *S. coelicolor chiF* shown by previous workers. A

common evolutionary origin of the enzyme was clearly indicated. As regards to the promoter sequence which governs the expression of the gene, the direct repeat sequence discovered at the promoter region (Figs. 6 and 7) was similar to that of *S. coelicolor chiF* (Saito *et al.*, 2000); its determinative regulatory role in substrate induction and glucose repression has been depicted (Ni and Westpheling, 1997). In a broth culture of *chiF* transformed *E. coli* Rosetta™ (DE3), the chitin induction and glucose repression of chiF production was demonstrated (data not shown). The promoter with alike direct repeat sequence was found not exclusive only for the family 19 chitinase gene targeted. We recently cloned a full length family 18 chitinase gene *chiA* from the test bacterium SGS3, sequence analysis has shown that it also harbors a direct repeat sequence of TGGTCTGGACCA at the promoter region (Chen *et al.*, unpublished). The *S. griseus* chiC enzyme purified from culture of *chiC* transformed *E. coli* was shown to be greatly inhibitory to the mycelial growth of *Trichoderma resii* (Watanabe *et al.*, 1999). For family 19 chitinase known among members of *Streptomyces*, the unique antifungal characteristics of the enzyme has been attributed mainly to the function of the catalytic domain and also, at least in part, its type 3 chitin binding domain (ChtBD). The type 3 ChtBD was shown consisting of 6 aromatic groups that appeared to be important in substrate binding (Watanabe *et al.* 1999). It was noted that the deletion of ChtBD might impair affinity of the enzyme to the substrate and thus greatly reduced the enzyme attributed growth inhibition (Itoh *et al.*, 2002; Tsujibo *et al.*, 2003). For SGS3 chiF, the chitin binding domain (ChtBD) located at the upstream of *chiF* cloned showed a CBM_5 12 carbohydrate binding domain characteristic and a type 3 ChtBD homologous AKWWTQ sequence identical to that known on *S. griseus chic* and *N. prasina chiB*. The enzyme purified from overexpression of the *chiF*- transformed *E. coli* Rosetta (DE3) broth culture was tested on plate culture against *Trichoderma virens*, *Alternaria* sp., *Rhizoctonia solani* AG4, *Rhizopus* sp. and *Pyricularia oryzae*. Although the sequence data of SGS3 *chiF* (Figs. 2 & 5) was highly identical to *S. griseus chiC* (87.4%) reported (Watanabe *et al.*, 1999), the antagonistic effect was not detected (data not shown). The discrepancy could be due mainly to the use of different microbial strains for gene expression and antagonistic bioassay. In a dual culture assay to illustrate the antifungal activity of SGS3 against various phytopathogenic fungi, remarkable mycoparasitic effect indicating clearly the functioning of chitinases(s) and/or glucanase(s) was consistently detected (data not shown). It seemed likely that the antifungal activity of SGS3 involved synergistic effect of cohorts of lytic enzymes rather than simply a single chiF.

The existence of family 19 chitinase has been shown by Watanabe *et al.*, (1999) a common characteristic among *Streptomyces* species. Since the initial discovery, family 19 chitinase gene was found also present in non-*Streptomyces* members such as *Burkholderia gladioli* (Kong *et al.*, 2001) and *Nocardiopsis prasina* OPC-131 (Tsujibo *et al.*, 2003). Moreover, the phylogenetic analysis of these microbial family 19 chitinase genes have suggested strongly that these genes in *Streptomyces* and *Burkholderia* might be acquired from plant source via horizontal gene transfer (Tsujibo *et al.*, 2003; Watanabe *et al.*, 1999). In the works herein presented, the detection of *chiF* by PCR amplification with the application of *chiF* specific primer +pair (Fig. 3) from most of the tested strains echoed their view in that the existence of family 19 chitinase was indeed a common characteristic among *Streptomyces* strains antagonistic to phytopathogenic fungi. For the 10 test strains which showed negative results in the performed PCR analysis, similar results were obtained in both northern analysis and southern analysis followed (data not shown). However, by western analysis, all these negative strains were detected positive the presence of chiF-antiserum reactive proteins (paper submitted). The immuno-detection results obtained further revealed that these enzyme proteins were serologically related and were mostly nearly identical in molecular weight. The close relevance suggested possibility that they might be evolved from a common phylogenetic origin.

ACKNOWLEDGEMENTS

This work was supported partly by a National Science Council research grant NSC92-2317-B005-013 and partly by a Council of Agriculture research grant COA92AS-1.4.1-ID-I1 from Executive Yuan, Taiwan, Republic of China.

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